



[haematologica reports]  
2006;2(7):27-29

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## Dissecting the natural history of chronic lymphocytic leukemia to discover new therapeutic targets

Chronic lymphocytic leukemia (CLL), the most frequent leukemia in the western world, cannot be cured with current treatment strategies. The central clinical problem of CLL is to define which patients should be treated, when and how. Dissecting the natural history of the disease may identify disease mechanisms which may prove to be of prognostic value or lead to define molecules of potential interest as treatment targets. The rationale of this approach is demonstrated by the observation that the analysis of the mutational status of the Variable genes of the Immunoglobulin (Ig) heavy chains (IgV<sub>H</sub>) has changed dramatically our perspective of CLL. IgV<sub>H</sub> gene analysis has shown that CLL consists of at least two disease subsets which can be distinguished by the incidence of somatic mutations in the IgV<sub>H</sub> genes. Importantly the prognosis of patients in the two subsets is markedly different, with those bearing unmutated VH genes being definitely worse.

Our strategy of investigation is based on the the three-step model of the natural history of CLL we have proposed, whereby in Step 1 unknown genetic abnormalities lead to the emergence of the transformed clonal founder cell, in Step 2 interactions with adequate microenvironments help the clonal progeny to avoid apoptosis and to progress and in Step 3 the accumulation of new and more dangerous genetic abnormalities favour the autonomous growth of malignant cells.

### **Differential effects on CLL cell survival are exerted by different microenvironmental elements**

All events that mark the natural history of CLL occur in tissues where the balance between proliferation and reduced apoptosis influences the lymphocyte accumulation. *In vivo* proliferation and extended survival are favoured by the leukemic cell capacity to respond to the proliferative and anti-apoptotic microenvironmental signals provided by tissue bystander cells through

cellular contacts and soluble factors.

Selected microenvironmental stimuli confer to leukemic cells a growth advantage and an extended survival. A key feature of CLL tissue microenvironment is the presence of Pseudofollicles (PF), clusters of proliferating blasts that are the disease cycling reservoir. CD4<sup>+</sup>T and stromal cells are invariably present within PF and appear to support the proliferation and prevent the apoptosis of malignant cells. To dissect the differential support provided by the different cellular components of the microenvironment where CLL cells accumulate, we cultured purified CLL cells *in vitro* in the presence or absence of different accessory cells (stromal cells, autologous T lymphocytes) and/or soluble molecules (IL-4, sCD40L) and assessed the leukemic cell response in terms of cell viability and chemoattracting capacity. The results indicate that both T lymphocytes and stromal cells are involved in sustaining the survival of leukemic B cells, but indicate that their support is different in terms of time of onset and duration. T cells have a *short term* support activity while stromal cells provide a *long term* support.

### **The issue of B-cell receptor (BCR)**

The presence of somatic mutations of IgV<sub>H</sub> genes indicates that, at least in a portion of cases, CLL cells had encountered an antigen during the natural history of the disease. Unmutated (U) cases show a remarkable skewing in IgV<sub>H</sub> gene usage. Through a large collaborative study we analyzed the IgHV repertoire and mutational status in 553 CLL patients from the Mediterranean area and showed that the most common IgHV genes are used in CLL at frequencies similar to those of normal B cells, except for the IgHV1-69 and the IgHV3-49 genes. We observed an underrepresentation of the IgHV1-18, IgHV3-30.3 and IgHV4-59 genes.

In addition, all CLL cases, both mutated (M) and U, show a common surface phenotype which is significantly activated and

similar to the surface phenotype of Ag-experienced B cells. The properties of CLL cell B-cell receptor (BCR) resemble those observed in normal B cells upon Ag interaction and gene profiling analyses revealed that both subsets share striking similarities with the so-called *memory B cells*. The detailed analyses of the Complementary Determining Regions 3 (CDR3) sequences of the leukemic Ig receptors showed that unrelated patients in different parts of the world express very similar if not identical B cell receptors (BCR). As an example, the IgHV3-21 gene, frequently expressed in Scandinavian CLL, is present only in a limited number of cases in the Mediterranean cohort studied. However, cluster analysis of HCDR3 in IgHV3-21-utilizing cases revealed the existence of a common-HCDR3 subset suggesting the existence of a potential antigenic element rarely encountered in the Mediterranean area. Remarkably, similar V<sub>H</sub>DJ<sub>H</sub> rearrangements have been identified in both U and M CLL, suggesting an antigenic selection in both subsets of the disease.

From all these evidences the concept has arisen that the cell of origin, regardless its mutational status, has to be an *antigen-experienced* B cell that gives rise to a malignant clone which appears to be more dynamic than previously appreciated and whose progression is favoured by a number of molecular and cellular interactions that occur in tissues.

#### **A proteomic investigation of the molecules involved in the signal transduction following stimulation of the BCR**

The precursor which underwent the first transforming event would differentiate into a mature B-cell that has a functional B-cell receptor (BCR) which may allow Ag intervention to trigger clonal expansion. Antigen encounter by the cell of origin is indicated in both CLL subsets (mutated and unmutated) by selective but distinct expression of V-genes, with evidence for continuing stimulation post-transformation. The key to distinctive tumor behavior likely relates to the differential ability of the BCR to respond to stimulation. Both subsets have been shown to undergo low level signaling *in vivo*, although analysis of blood cells limits knowledge of critical events in tissues. Analysis of signal competence *in vitro* reveals that unmutated CLL continues to respond, whereas mutated CLL is anergized. Differential responsiveness may reflect the increased ability of post-germinal center B cells to be triggered by antigen, leading to long-term anergy. This could minimize division in mutated CLL and account for prognostic differences.

We have pursued the molecular and functional characterization of the signal transduction molecules involved in the two subsets of patients by proteomic

technologies. The aim has been to visualize both quantitative and qualitative (post-translational modifications) differences in protein expression and to compare the proteomic profiles of the different clinical and biological CLL subsets. This has focussed our attention upon two regions of interest of the proteomic maps which we have analyzed by mass spectrometry (MS). The first region of interest corresponds to hematopoietic-lineage-cell-specific-protein-1 (HS1), a protein pivotal in the signal cascade triggered by the BCR stimulation. In bad prognosis patients most HS1 protein is constitutively phosphorylated, whereas only a fraction is phosphorylated in good prognosis patients. The survival curve of all 60 cases analyzed reveals that patients with prevalently phosphorylated HS1 have a significantly shorter median survival time ( $p=0.015$ ). The pattern of HS1 expression following BCR engagement in normal mature B-cells stimulated by anti-IgM reveal a shift of the non- or less-phosphorylated form of HS1 toward the more phosphorylated form. Naive B-cells show both HS1 forms while memory B-cells expressed mainly the phosphorylated fraction.

These data suggest that HS1 may prove to be a new therapeutic target for patients with aggressive disease. The function of HS1 is presently unknown. The analysis of the amino acid sequence is not unequivocal, suggesting either an actin-binding activity or a signal transduction function. Confocal microscopy and co-immunoprecipitation experiments with antibodies against cytoskeleton components are revealing potential interactions.

A second region of interest involves two other differentially expressed spots that we have both identified by MS as Glyoxalase I (Glu1). Again, there are no differences in the amount of protein expressed in all CLL cases, while the presence of single or double spots due to a conformational change appeared to discriminate patients in terms of response to therapy. This might be related to the fact that Glu1 plays a critical role in the detoxification of dicarbonyl compounds and is found in drug-resistant tumors cells.

Finally, another molecules involved in lymphocyte signaling pathways is the tyrosine kinase ZAP-70 originally described only in T cells and natural killer cells. In CLL ZAP-70 expression correlates with clinical outcome. ZAP-70 was analyzed in several B-cell lines, *ex vivo* malignant B-cells, ranging from acute lymphoblastic leukemia to multiple myeloma, as well as in different human normal B lymphocyte subpopulations: naive, germinal-center and memory B-cells from tonsils, CD19+ CD5+ cells from cord blood and CD19+ lymphocytes from peripheral blood. All expressed ZAP-70 protein, though at different levels depending on their differentiation, activation and tissue localization. In addition, Zap-70 expression levels could be modu-

lated following stimulation via the BCR. These findings implicate a potential role for ZAP-70 in the signaling pathway of B lymphocytes at different maturational stages.

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